



## **Genome-wide targets of selection: female response to experimental removal of sexual selection in *Drosophila melanogaster***

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bioRxiv first posted online November 12, 2013

Access the most recent version at doi: <http://dx.doi.org/10.1101/000240>

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1 Genome-wide targets of selection: female response to experimental  
2 removal of sexual selection in *Drosophila melanogaster*

3

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11 short running title: Genome-wide targets of sexual selection

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18

**19 ABSTRACT**

20 Despite the common assumption that promiscuity should in general be favored  
21 in males, but not in females, to date there is no consensus on the general impact  
22 of multiple mating on female fitness. Notably, very little is known about the  
23 genetic and physiological features underlying the female response to sexual  
24 selection pressures. By combining an experimental evolution approach with  
25 genomic techniques, we investigated the effects of single and multiple matings  
26 on female fecundity and gene expression. We experimentally manipulated the  
27 mating system in replicate populations of *Drosophila melanogaster* by removing  
28 sexual selection, with the aim of testing differences in short term post-mating  
29 effects of females evolved under different mating strategies. We show that  
30 monogamous females suffer decreased fecundity, a decrease that was partially  
31 recovered by experimentally reversing the selection pressure back to the  
32 ancestral promiscuous state. The post-mating gene expression profiles of  
33 monogamous females differ significantly from promiscuous females, involving  
34 9% of the genes tested. These transcripts are active in several tissues, mainly  
35 ovaries, neural tissues and midgut, and are involved in metabolic processes,  
36 reproduction and signaling pathways. Our results demonstrate how the female  
37 post-mating response can evolve under different mating systems, and provide  
38 novel insights into the genes targeted by sexual selection in females, by  
39 identifying a list of candidate genes responsible for the decrease in female  
40 fecundity in the absence of promiscuity.

41

42 Keywords: Sexual selection, experimental evolution, transcriptomics, mating  
43 systems, female postmating response.

44

45

46 **Introduction**

47 The evolution of mating strategies, and in particular of female polyandry, has  
 48 attracted a great deal of attention in recent decades (Andersson, 1994; Birkhead,  
 49 2000; Shuster & Wade, 2003). The debate on the adaptive significance of female  
 50 multiple mating stems from the common assumption that males exhibit a  
 51 stronger positive covariance between promiscuity and reproductive success  
 52 than females. In other words, males may gain more offspring by repeated  
 53 matings than females, even though both sexes have the same average numbers of  
 54 matings, mates and offspring (Shuster & Wade, 2003). Polyandry is also assumed  
 55 to carry costs in terms of time and energy for additional matings (Jormalainen *et al.*,  
 56 2001; Wedell *et al.*, 2006) or physical injury (Boeuf & Mesnick, 1991; Hurst *et al.*,  
 57 1995), as well as an increased risk of predation (Wing, 1988) and infection  
 58 during copulation (Hurst *et al.*, 1995).

59

60 Empirical studies however, show that in the vast majority of species, females  
 61 often mate with more than one male (Ridley, 1988; Andersson, 1994; Simmons,  
 62 2001). Theoretically, female polyandry can be promoted by selection if males  
 63 provide resource benefits, through the ejaculate (Thornhill, 1976; Boggs &  
 64 Gilbert, 1979; Turner & Anderson, 1983) or through additional paternal care  
 65 (Stacey, 1982; Davies, 1985), or if some males do not provide viable sperm or  
 66 insufficient ejaculate to fertilize the ova (Gibson & Jewell, 1982; Gromko *et al.*,  
 67 1984). It has also be proposed as a strategy to reduce sexual harassment (Svärd  
 68 & Wiklund, 1986). Moreover, there could be indirect genetic benefits by  
 69 acquiring 'good genes', compatible genes or in producing genetically diverse  
 70 progeny or promoting sperm competition (Yasui, 1998). Finally, multiple mating  
 71 can be non-adaptive for females in the presence of strong selection for multiple  
 72 mating on males coupled with a strong intersexual genetic correlation for mating  
 73 propensity (Halliday & Arnold, 1987).

74

75 Recently, experimental evolution studies have been an increasingly popular  
 76 approach to evaluate the effect of different mating systems on male or female  
 77 fitness (Edward *et al.*, 2010), predominantly within the framework of sexually

antagonistic co-evolution. Under a promiscuous mating system, where the fitness values of an individual and its mate are not perfectly correlated, pre-copulatory and post-copulatory intrasexual competition are expected to result in the evolution of traits that increase the reproductive success of members of one sex at the expense of the other, in a co-evolutionary arms race called interlocus sexual conflict (Parker, 1979). An eminent example of harm induced by males to females in an attempt to maximize their mating rate and fertilization success is represented by *Drosophila melanogaster*, in which courtship and transfer of seminal fluid are known to increase female mortality rate and decrease lifetime reproductive success while increasing male competitive abilities (Chapman *et al.*, 1995).

Holland and Rice (1999) were the first to investigate the change in female reproductive success in populations of *D. melanogaster* using an experimental evolution design where sexual selection was removed by enforcing monogamy and random mating assignment. They found that monogamous populations had greater net reproductive rate than (promiscuous) controls, while fecundity of monogamous females was reduced after mating with ancestral (promiscuous) males (Holland & Rice, 1999). After this seminal paper, several other studies employed a similar methodology in different taxa (Hosken *et al.*, 2001, 2009; Martin & Hosken, 2003; Crudgington *et al.*, 2005; Tilszer *et al.*, 2006; Bacigalupe *et al.*, 2007; Fricke & Arnqvist, 2007; LaMunyon *et al.*, 2007; Simmons & Garcia Gonzalez, 2008; Gay *et al.*, 2009; Maklakov *et al.*, 2009), briefly reviewed by Edward *et al.* (2010), with some degree of variation in experimental design and outcome.

Regardless of the adaptive significance of female polyandry, the genetic basis of the fitness components that depend on different mating strategies is a key aspect, which has so far received little attention. In other words, very little information is available about the characteristics and identity of the genes that respond to an alteration of sexual selection (i.e. the targets of selection, but see Immonen & Ritchie, 2011). With modern genomic techniques, it is possible to scan whole genomes and transcriptomes and associate them with the

111 corresponding phenotypes. Coupling experimental evolution with genome  
 112 sequencing or transcriptome profiling is a very recent and successful approach  
 113 (Burke *et al.*, 2010; Zhou *et al.*, 2011), in that it experimentally magnifies the  
 114 variation in the trait of interest and produces a greater resolving power in  
 115 identifying structure of molecular networks and adaptive processes (Turner *et*  
 116 *al.*, 2011). However, these methods to our knowledge have not been applied to  
 117 sexual selection studies so far.

118  
 119 Conversely, other aspects of the fruit fly reproductive biology are much better  
 120 known. In recent years, considerable quantities of data have been collected on  
 121 the female physiological changes associated with the shifts in female mating  
 122 status. Molecular and genomics techniques have been employed to investigate  
 123 the effects of mating in *D. melanogaster* (Lawniczak & Begun, 2004; McGraw *et*  
 124 *al.*, 2004, 2008; Mack *et al.*, 2006; Innocenti & Morrow, 2009). In particular,  
 125 several detailed studies have focused on seminal fluid components on female  
 126 post-mating physiology, leading to the identification of several seminal fluid  
 127 proteins (SFP) and to the isolation of their effect in females (reviewed in Ravi  
 128 Ram & Wolfner, 2007; Avila *et al.*, 2011), including the characterization of sex-  
 129 peptide and its receptor (Domanitskaya *et al.*, 2007; Yapici *et al.*, 2008).

130  
 131 Here, we integrate these approaches to investigate the evolutionary response of  
 132 populations experiencing differing sexual selection pressures, at both a  
 133 phenotypic and genomic level, allowing a direct comparison between the two.  
 134 We begin by using experimental evolution to evaluate the effects of the removal  
 135 of components of sexual selection in a laboratory-adapted population of *D.*  
 136 *melanogaster*. The effects of enforced monogamy are then investigated both in  
 137 terms of differences in female reproductive output and in female post-mating  
 138 response, measured as genome-wide gene expression profiles. In addition, for  
 139 populations that have evolved under enforced monogamy we subsequently  
 140 reverse the selection pressure back to the ancestral promiscuous state and again  
 141 investigate how reproductive output is affected. We take an exploratory  
 142 approach to investigate the characteristics and biology of those transcripts  
 143 identified as being influenced by the experimental selection regimes, with the

ultimate aim of understanding in more detail which biological processes in females are associated with evolutionary changes in mating system.

## Materials and Methods

### *Fly stocks*

All flies used to constitute the experimental evolution lines were derived from a large outbred wild-type population of *D. melanogaster* (LH<sub>M</sub>) that had been maintained under the same rearing protocol for over 400 non-overlapping generations (for a detailed description, see Rice *et al.*, 2005). The population is maintained in a set of 56 vials at a large size (1792 adults) under competitive conditions and at moderate larval density in standard rearing environment: 25°C, cornmeal/molasses/yeast/agar medium, 12h:12h light/dark cycle, 16 individuals of each sex per vial (25mm x 95 mm) with a 14 day generation cycle. We applied the same culturing condition to our experimental lines, unless otherwise specified.

### *Experimental evolution lines*

In March 2008, a replicate of the ancestral LH<sub>M</sub> population was obtained by allowing females to lay eggs for 18 h (Day 0). On the day of emergence, Day 10, we collected 384 virgin males and 384 virgin females from the base population, and randomly assigned them to 2 treatments, each constituted by 4 replicate populations of 96 individuals, and stored separately by sex. On Day 13, males and females were placed together in fresh vials (16 pairs per vials, three vials per population) with 6 mg fresh yeast and allowed to mate. In one treatment (hereafter referred to as “monogamous treatment”), males were removed after 1 h under brief CO<sub>2</sub> anesthesia and discarded. During this window of time, in our LH<sub>M</sub> population virtually all the sexually mature and healthy females mate, but none of the females mate twice, due to their refractory period. We performed a preliminary study using time-lapse photography (see Kuijper & Morrow, 2009) to confirm this pattern: we placed 25 vials containing 10 virgin males and 10 virgin females in an incubator under standard conditions and monitored their activity for 12 h. The results show a peak in mating activity (often 10 pairs simultaneously) between 10 and 30 minutes, followed by a long (>1 h)

177 refractory period, after which females start re-mating (Fig. S8). In the other  
 178 treatment (hereafter “promiscuous treatment”), males were left in the vials with  
 179 the females and allowed to mate further. On day 14 (i.e. Day 0 of the following  
 180 generation), the flies were transferred to fresh vials to oviposit for 18 h. The  
 181 following day (Day 1), eggs were counted and those exceeding 150 were  
 182 removed to ensure a uniform larval environment. On Day 10, 96 individuals per  
 183 replicate population were collected as virgin (48 males and 48 females) and the  
 184 same culturing conditions described above were applied every generation.

185

#### 186 *Body size*

187 After 30 generations of experimental evolution we harvested 40 males and 40  
 188 females from each replicate population to assess whether there had been a  
 189 change in body size. A single wing was removed from each individual, mounted  
 190 on a slide using transparent tape and photographed using bright field-  
 191 illumination (x40 magnification). Length was measured using the straight line  
 192 tool in ImageJ (Rasband, 1997), from the intersection of the anterior cross vein  
 193 and longitudinal vein 3 (L3) to the intersection of L3 with the distal wing margin  
 194 (Partridge *et al.*, 1987).

195

#### 196 *Female fecundity*

197 The effect of the treatment on female fecundity was assayed with a factorial  
 198 design in four different trials, after 30, 31, 50 and 58 generations of experimental  
 199 evolution, with slightly different experimental designs (trials 1-4 respectively,  
 200 see below). For each trial, the following protocol was applied: on day 14 of the  
 201 chosen generation, a replicate of the experimental lines were obtained, by  
 202 allowing flies to oviposit for an additional 24 h in fresh vials. The populations  
 203 obtained were cultured with standard protocol (i.e. as in the promiscuous  
 204 treatment) for a generation to remove parental effects. On Day 10 of the  
 205 following generation, 160 females and 160 males for each treatment and  
 206 replicate were collected as virgins and stored separately (10 vials of males and  
 207 10 vials of females for each of the 8 experimental lines). On Day 13, half of the  
 208 females from each experimental line (5 vials) were crossed to males from the  
 209 same experimental line, and the other half (5 vials) were crossed to males from a



single replicate population of the other treatment (females from replicate 1 of the monogamous treatment were mated to males from replicate 1 of the promiscuous treatment, replicate 2 of the monogamous treatment was paired with replicate 2 of the promiscuous treatment, and so on), and allowed to mate in fresh vials containing 6 mg live yeast. At this stage, the trials differed in their design, as follows.

216

In trial 1, males were removed and discarded the following day (after 30 h, Day 14), while individual females were transferred in oviposition test tubes, and allowed to oviposit for 18 hours, corresponding to the window of time in which eggs laid by females in the experimental populations were retained for the next generation. Females were then discarded, the tubes refrigerated for 24 hours and the eggs counted. In trial 2, the protocol employed was identical to the one described for trial 1, except the males were removed and discarded after 1 h, allowing females to mate only once.

225

In trial 3, after 1 h, all the males were removed and discarded. Females were allowed to oviposit, and were transferred every 12 h (at 9:00 and at 21:00) to a fresh vial for four days (6 times, 7 time-points) to avoid excessive larval density, then discarded. When the new generation emerged, progeny were counted. In trial 4, the protocol employed was very similar to the one described for trial 3, with the following differences: after crossing target males and females, males were not removed from the vials; also, during the four days of oviposition the flies were transferred every 6 h during the daylight hours (9:00, 15:00 and 21:00; 9 times, 10 time-points).

235

In summary, we obtained measures of fecundity of females in our experimental lines under four different conditions: after a single mating and after being continuously exposed to males, during the whole period in which they were allowed to oviposit in our selection regime, or during a longer timeframe, to account for potential shifts in the resource that females allocate to eggs over time.

242

243 *Reversed selection lines*

244 After 95 generations of experimental evolution a third treatment was established  
245 using surplus flies harvested from each of the 4 monogamous populations. In this  
246 new treatment, the rearing protocol was identical to that for the promiscuous  
247 treatment, and therefore flies in these populations experienced a reversal of the  
248 selection pressure from a monogamous to a promiscuous mating system  
249 (hereafter refereed to as the MP treatment). After a further 25 generations of  
250 experimental evolution (generation 120 in total) the populations were cultured  
251 again with standard protocol for a single generation to remove parental effects,  
252 then an assay of female fitness from all replicate populations and treatments was  
253 performed (n=53-64 individual females per population), using the same protocol  
254 employed for trial 1 (see above).

255

256 *Microarray data*

257 After 46 generations, on day 14, replicates of the experimental lines were  
258 obtained, by allowing flies to oviposit for additional 24 h in fresh vials. The  
259 populations obtained were cultured with standard protocol (i.e. as the  
260 promiscuous treatment) for a generation to remove parental effects. On Day 10  
261 of the second generation, 64 females and 64 males for each treatment and  
262 replicate were collected as virgins and stored separately (4 vials of males and 4  
263 vials of females for each of the 8 experimental lines). On Day 13, half of the  
264 females from each experimental line (2 vials) were crossed to males from the  
265 same experimental line, and the other half (2 vials) were crossed to males from a  
266 single replicate population of the other treatment, and allowed to mate in fresh  
267 vials containing 6 mg live yeast. After 1 h, all the males were removed and  
268 discarded, while the females were randomly divided in two groups of 8 flies  
269 under brief CO<sub>2</sub> anesthesia, to be used as a main sample and its backup. After 6  
270 hours, the females were flash-frozen in liquid nitrogen and stored at -80°C for no  
271 more than four days until RNA extraction. Hence, for each of the replicate  
272 population we collected 4 independent samples of eight females, two samples of  
273 females mated to males of the same replicate population, and two samples of  
274 females mated to males of a single replicate population in the other treatment,  
275 giving a total of 32 samples. Total RNA was extracted independently from each

sample using Trizol (Invitrogen, Carlsbad, CA, USA) and purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quality and quantity was assessed with an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA samples were prepared and hybridized to Affymetrix Drosophila GeneChip 2.0 microarrays at the Uppsala Array Platform (Uppsala, Sweden) following manufacturer's instructions. The arrays were scanned in two batches of 16, balanced for replicate population of origin and replicate population of origin of the males to which they were mated.

284

#### 285 *Statistical analysis*

286 All statistical analyses were run in the R environment (version 2.11.1 for most  
287 analyses, version 3.0.1 for body size and reversed experimental evolution assay,  
288 available at [www.r-project.org](http://www.r-project.org) R Development Core Team, 2009).

289

290 Male and female body size was analyzed using a full factorial linear model (lm  
291 function; mating system and sex as fixed effects) using within replicate means to  
292 avoid pseudoreplication (n=36).

293

294 Female fecundity data for each trial were analyzed using linear models (lm  
295 function). In all cases, amount of eggs or progeny produced was averaged within  
296 replicate population and summed across time points (for trials 3 and 4), to avoid  
297 pseudoreplication. We fitted the following model to each dataset:

298

299

$$300 \quad y_{ijk} = f_i + m_j + I_{ij} + e_{ijk}$$

301

302 with  $i = \{1,2\}$ ;  $j = \{1,2\}$ ;  $k=\{1,...,4\}$ ; where  $y$  is the number of progeny/eggs  
303 produced by females after each cross,  $f$  is the treatment of origin of the females  
304 (monogamous or promiscuous, fixed effect),  $m$  is the treatment of origin of the  
305 males to which females were mated (monogamous or promiscuous, fixed effect)  
306 and  $I$  is their interaction. The interaction term was subsequently dropped,  
307 because it was not significant in any trial and did not improve the fit of the  
308 models ( $P>0.25$  for all models).

309  
 310 Microarray data were analysed using the BIOCONDUCTOR suite of packages  
 311 (Gentleman *et al.*, 2004) in R. To pre-process the raw expression data, we used  
 312 the standard RMA (Robust Multichip Average) algorithm (Irizarry *et al.*, 2003)  
 313 implemented in the AFFY package (Gautier *et al.*, 2004). After pre-processing the  
 314 resulting dataset was filtered to exclude features according the following  
 315 criteria: (i) probe sets without an Entrez Gene ID annotation, (ii) Affymetrix  
 316 quality control probe sets, (iii) if multiple probe sets mapped to the same Entrez  
 317 Gene ID, only the probe set with the highest coefficient of variation was retained.  
 318 Out of the original 18952 features, the filtering step removed 6380 probe sets,  
 319 while 12572 probe sets, corresponding to as many known genes, were retained  
 320 for the statistical analyses.

321  
 322 Significance of differential expression was assessed using the package LIMMA  
 323 (Linear Models for Microarray Data; Smyth, 2005). A model matrix was designed  
 324 to fit a parameter for every combination of replicate population of origin of  
 325 females (n=8) and population of origin of males to which females were mated  
 326 (n=8), for a total of 16 parameters. An additional random effect with two levels  
 327 was fitted to control for the batch effect, and estimated borrowing information  
 328 between features, by constraining the within-block correlations to be equal  
 329 across features and by using empirical Bayes methods to moderate the standard  
 330 deviations (Smyth, 2005). A contrast matrix was designed to obtain the contrasts  
 331 of interest: the main effect of treatment of origin of females, the main effect of  
 332 treatment of origin of males to which females were mated, and their interaction.  
 333 All the resulting P values were corrected for multiple testing to obtain a  
 334 maximum false discovery rate of 5% (FDR; Benjamini & Hochberg, 1995;  
 335 corrected  $P < 0.05$ ).

336  
 337 We used a mean-rank gene set enrichment test (MR-GSE test, implemented in  
 338 LIMMA; Michaud *et al.*, 2008) to test whether the sets of up-regulated or down-  
 339 regulated significant transcripts showed a tendency to be up- or down-regulated  
 340 after mating, using the *t*-values from a contrast between virgin and mated  
 341 females from the same population, from a previously published study (Innocenti

342 & Morrow, 2009). We defined as 'Virgin-like' the subset of transcripts for which  
 343 the expression in the monogamous female is lower than the promiscuous  
 344 females if the gene is up-regulated by mating, or higher if the gene is down-  
 345 regulated by mating; in other words, genes whose profile is more similar to a  
 346 virgin fly. 'Mated-like' genes are the complimentary set of genes. A MR-GSE test  
 347 was also used to test whether the set of significant transcripts showed a  
 348 tendency to be associated with female fitness, using the  $t$ -values from a  
 349 previously published study (Innocenti & Morrow, 2010) on the same population.

350

351 Among the genes found to be differentially expressed, we identified  
 352 transcriptional modules of correlated expression across-tissues using the HOPACH  
 353 package (Hierarchical Orderer Partitioning and Collapsing Hybrid; van der Laan  
 354 & Pollard, 2003). We computed a distance matrix using the pairwise correlations  
 355  $r_{ij}$  between the expression of the significant transcripts across different tissues of  
 356 *D. melanogaster*. The tissue-specific expression data were produced by the  
 357 FlyAtlas team (Chintapalli *et al.*, 2007), available on the Gene Expression  
 358 Omnibus (GEO) database with accession number GSE7763, and normalized  
 359 according to a method described elsewhere (Innocenti & Morrow, 2010). The  
 360 clustering algorithm built a hierarchical tree by recursively partitioning or  
 361 collapsing clusters at each level, using MSS (Median Split Silhouette) criteria to  
 362 identify the level of the tree with maximally homogeneous clusters (van der Laan  
 363 & Pollard, 2003).

364

365 We selected the modules containing more than 50 genes (the clusters more  
 366 likely to provide biologically meaningful within-module summary statistics) and  
 367 analyzed them to identify whether they showed: (i) association with genes  
 368 involved in female post-mating response (see above); (ii) association with female  
 369 fitness (see above); (iii) non random chromosomal distribution; (iv) over-  
 370 represented Gene Ontology categories; (v) tissue enrichment or specificity. Non-  
 371 random chromosomal distribution was assessed with a Fisher's exact test on the  
 372 expected and observed number of genes on each chromosome ( $P < 0.01$ ). To  
 373 identify GO categories enriched for particular subsets of transcripts, we used a  
 374 hypergeometric test for over-representation ( $P < 0.01$ , GOSTATS package; Falcon

& Gentleman, 2007). We identified tissue-enriched or tissue-specific transcripts using data from the FlyAtlas database (described above). The tissue specific expression levels for the list of transcripts in each module were obtained, and the modules were tested for over-abundance of genes of interest in a target tissue using a one-tailed Fisher's exact test. All the reported P values were Bonferroni corrected for testing on multiple tissues ( $P < 0.01$ ,  $n = 17$ ).

## Results

### *Body size*

After 30 generations of experimental evolution the body sizes of individuals from the two selection regimes remained virtually unchanged, with males and females of promiscuous lines having approximately 1% smaller wings than those in monogamous lines (mating system effect:  $F_{3,12} = 1.84$ ,  $P = 0.200$ ; Fig. 1).

### *Female fecundity*

The reproductive output of promiscuous females was greater than those of monogamous females, regardless of the males they were mated with (Fig. 2; see Material and Methods). This difference was significant when measured as number of eggs laid in a 18 h period, corresponding to the oviposition period in every generation of experimental evolution (as well as the ancestral population), both after a single mating with a male ( $F_{1,13} = 4.89$ ,  $P = 0.046$ ), or being continuously exposed to males ( $F_{1,13} = 11.32$ ,  $P = 0.005$ ). When measured as number of adult progeny emerging from eggs laid during a period of 4 days, this difference was significant only after a single mating ( $F_{1,13} = 6.06$ ,  $P = 0.029$ ), but not when females were continuously exposed to males ( $F_{1,13} = 2.93$ ,  $P = 0.110$ ), although the effect sizes were comparable for direction and magnitude (Table 1).

### *Reversed selection lines*

As described in the Material and Methods, the reversed selection lines (MP) were established after the monogamous and promiscuous populations had already undergone 95 generations of selection. All three treatments were then run for a further 25 generations prior to the final assays of fecundity being performed. Despite this substantial additional period of experimental evolution, relative to

the first round of assays (a further 90 generations), the patterns of reproductive output between monogamous and promiscuous females was remarkably similar at these two time points (M vs P differences: G30 45%; G120 36%), indicating that the majority of phenotypic evolution had occurred within the first 30 generations (posthoc Tukey HSD: M vs P,  $t = 2.716$ ,  $P=0.0279$ ; Fig 3). The phenotypic change in reproductive output of females from the MP populations following 25 generations of reversed selection was smaller. It did however indicate a reversal in reproductive output had occurred; posthoc tests showed that the reproductive output of females from the MP treatment was intermediate to both monogamous and promiscuous treatments (Tukey HSD: M vs MP,  $t = 0.913$ ,  $P=0.6365$ ; MP vs P,  $t = 1.804$ ,  $P=0.1850$ ; Fig. 3).

419

#### 420 *Gene expression profiles*

After 46 generations of selection, we tested the difference in female genome-wide post-mating response, by measuring gene expression in adult *D. melanogaster* females evolved under different sexual selection regimes (monogamous and promiscuous). After multiple testing correction, monogamous and promiscuous females showed a significant difference in the expression of 1141 transcripts ( $\approx 9\%$  of the transcripts analyzed, at 5% F.D.R.), while male type and the interaction of female type and male type did not significantly affect the post-mating expression patterns. Among the differentially expressed transcripts, 438 were up-regulated and 703 down-regulated in monogamous females (Binomial test: ratio=0.38,  $P<0.0001$ ).

431

We compared the expression profile of these transcripts with the female post-mating response characteristic to the ancestral population (Innocenti & Morrow, 2009), and found that the expression level of 728 genes is altered in monogamous females to a lesser extent after mating, compared to promiscuous females (hereafter 'virgin-like', see Material and Methods), while the post-mating reaction of the remaining 413 genes is altered to a higher extent in monogamous females compared to promiscuous females (hereafter 'mated-like'), and their proportion is higher than expected by chance (Binomial test: ratio=0.64,  $P<0.0001$ ). In general, genes that are down-regulated in monogamous vs.

441 promiscuous mated females tend to be switched on (up-regulated) by mating  
 442 (two-tailed MR-GSE test:  $P < 0.0001$ , Fig. 4A) and genes that are up-regulated in  
 443 monogamous vs. promiscuous mated females tend to be switched off (down-  
 444 regulated) by mating (two-tailed MR-GSE test:  $P < 0.0001$ , Fig. 4A). We also used  
 445 previously published and independently derived data (Innocenti & Morrow,  
 446 2010) to test the relationship between the significant transcripts identified in  
 447 this study and female fecundity, and found them to be over-represented among  
 448 genes strongly associated with female fitness (irrespective of the sign of the  
 449 association; MR-GSE test,  $P < 0.0001$ , Fig. 4B).

450

451 In order to identify clusters of transcripts co-expressed in one or more tissues,  
 452 and hence possibly involved in similar biological function, we calculated modules  
 453 of correlated expression among the significant transcripts using data from the  
 454 FlyAtlas database (Chintapalli *et al.*, 2007). Among them, we selected the 7  
 455 clusters containing more than 50 genes (Fig. 5), which represented about 75% of  
 456 the significant transcripts, and evaluated their post-mating expression profile in  
 457 comparison to the ancestral population, their tissue specificity, chromosomal  
 458 distribution and over-presentation among Gene Ontology categories (see  
 459 Supplementary Information).

460

461 Module 1 contains genes highly specific for the male gonads, showing little or no  
 462 expression in other tissues (Fig. S1D,E). Overall, they do not tend to be perturbed  
 463 by mating (Fig. S1A). Over-represented Gene Ontology (GO) terms indicate that  
 464 the activity of a portion of these genes is linked to mitochondrial cellular  
 465 components (cellular respiration, electron transport, Table S1).

466

467 Module 2 is a large cluster of genes active in the majority of the tissue types (but  
 468 generally not in the gonads, Fig. S2E), and significantly over-expressed in the  
 469 head, eyes, carcass, fat body, heart and spermatheca. These transcripts are  
 470 subject to changes in expression levels after mating (Table 2), with mated  
 471 monogamous flies showing a more virgin-like expression profile for these genes  
 472 (Fig. S2A). They are chiefly involved in enzymatic metabolic activity (oxidation



473 reduction, proteolysis, Table S2). The left arm of chromosome 2 is enriched for  
 474 this set of genes (Fisher exact test: Odds-ratio=1.49, P=0.005).

475

476 The activity of genes clustered in module 3 is very similar to those of module 2:  
 477 these transcripts are active ubiquitously in the fruit fly tissues (Fig. S3D,E) and  
 478 the up-regulated subset is significantly enriched among the set of genes which  
 479 respond to mating (Table 2). Although not significant under our cut-off, a higher  
 480 than expected proportion of these genes lies on chromosome 2L (Odds-  
 481 ratio=1.47, P=0.045). GO terms associated with these genes include, again, strong  
 482 cytoplasmic enzymatic activity (oxidation reduction, catalytic activity, Table S3).

483

484 Module 4 presents the most distinctive and peculiar patterns. The majority of  
 485 these genes are down-regulated in the monogamous treatment (91 out of 124,  
 486 Table 2) and tend to be strongly affected by mating and distinctly more virgin-  
 487 like in monogamous females (Fig. S4A). These transcripts are consistently highly  
 488 expressed in the midgut, but relatively silent in all the other tissues (Fig. S4D,E),  
 489 and most of their activity is linked to metabolic processes, mainly peptidase and  
 490 hydrolase activity (Table S4). The distribution on the chromosomes is  
 491 significantly skewed towards the right arm of chromosome 2 (Odds-ratio=1.49,  
 492 P=0.009).

493

494 Modules 5 and 6 show highest relative expression levels in the ovaries, although  
 495 the transcripts are also active at slightly lower levels in every other tissue. These  
 496 genes tend to be overall weakly down-regulated after mating (Figs. S5A and  
 497 S6A). Module 5 showed relative virgin-like expression in monogamous females  
 498 compared to promiscuous females (Table 2). Perhaps unsurprisingly, sexual  
 499 reproduction and female gamete generation were among the most enriched  
 500 biological processes, while the same sets of genes were linked to nucleic acid and  
 501 protein binding molecular functions (Table S5). Module 6, also significantly over-  
 502 expressed in the brain, showed enrichment for biological processes such as  
 503 behaviour and signaling processes (Table S6).

504

Module 7 contains genes significantly more active in neural tissues: brain, thoracic ganglion, head and eyes (Fig. S7D,E). They are mostly down-regulated (Table 2) in monogamous females, but tend to be up-regulated after mating (virgin-like in monogamous females, Fig. S7A).

All modules tend to be associated with female fitness, with transcripts in modules 2, 3 and 4 showing the strongest association (Table 2).

## Discussion

In our study, we experimentally manipulated the mating system in replicate populations of *D. melanogaster*, by removing sexual selection, with the aim of testing differences in short term post-mating reaction of females evolved under different mating strategies. We showed that monogamous females suffer decreased fecundity, regardless of the type of male they were mated with, or whether mated once or continuously exposed to males. We also showed that monogamous females could recover some of this loss in fecundity if the selection pressure was reversed experimentally. Previously, Holland and Rice (1999) removed sexual selection in experimental lines from the same population (LHM) by manipulating sex ratio, and found that (i) monogamous females showed higher 'net reproductive rate' (female fecundity and offspring survival) than controls when mated with males from their own populations, and (ii) monogamous females showed lower fecundity than controls when mated once to ancestral (promiscuous) males (Holland & Rice, 1999). Monogamous males, in turn, evolved decreased courtship rate. Our experiment employed a different design, which allowed mass mating (mate choice and pre-copulatory intra-sexual competition) but a single mating event in the monogamous treatment, in order to leave selection on courtship rate unaffected. Our results showed no effect of male type on female fecundity (and consequently no interaction between male and female type), which rules out the possibility that males evolved decreased courtship intensity or a less harmful ejaculate. It is thus unlikely that the decrease in fecundity of monogamous females reflects a selective pressure towards less 'resistant' females. On the other hand, the experimental treatment removed continuous male harassment in the monogamous environment and

538 decreased population density during selection. Relaxed selection on resistance to  
 539 male harassment may have allowed the accumulation of deleterious mutations  
 540 or recombination of extant genetic variation with sub-optimal epistatic effects  
 541 which resulted in overall decrease in mean female fitness in the monogamous  
 542 environment. The decline in female fitness in monogamous lines is not likely to  
 543 be due to simple differences in population size and subsequent inbreeding, since  
 544 not only does theoretical and previous empirical work indicate that  $n = 96$  is  
 545 above the threshold for drift decay (see Morrow *et al.*, 2008), our reversed  
 546 experimental evolution treatment showed that the significant differences in  
 547 fecundity between monogamous and promiscuous females disappeared when  
 548 monogamous females experienced a reintroduction of a promiscuous mating  
 549 system. Such a response would not occur if monogamous populations had simply  
 550 become bottlenecked. This is further supported by the minimal differences in  
 551 body size seen between monogamous and promiscuous treatments, a trait that  
 552 could be sensitive to inbreeding.

553  
 554 The results of our genome-wide expression analysis confirmed a significant  
 555 difference between post-mating reaction between monogamous and  
 556 promiscuous females, while the evolutionary history of the males to which they  
 557 were mated did not influence their expression profiles. The genes that evolved to  
 558 respond differently to mating accounted for around 9% of the transcriptome  
 559 tested. When comparing transcriptional changes which occur when a female  
 560 switches between the virgin and mated status with differential expression  
 561 between mated monogamous and promiscuous females, it is clear that genes  
 562 which are up-regulated by mating tend to be down-regulated in monogamous vs.  
 563 promiscuous females (and those down-regulated by mating tend to be up-  
 564 regulated in monogamous vs promiscuous females; Fig. 4A), i.e. monogamous  
 565 females generally show a more 'virgin-like' expression profile. Similarly,  
 566 significant genes tend to be over-represented among candidate genes known to  
 567 be associated with female fitness. Taken together, these two lines of evidence can  
 568 be interpreted as strong support for the phenotypic results showing decreased  
 569 female fecundity: monogamous females seem to exhibit a weaker post-mating

570 reaction, both in terms of the extent to which genes are expressed and how many  
 571 eggs they lay.

572

573 When analysing and partitioning these genes according to the tissue where they  
 574 are predominantly active, we can identify 4 broad categories: transcripts active  
 575 in i) the midgut, ii) the ovaries, iii) neural tissues and iv) a wide range of tissues.  
 576 The midgut (module 3, Table 2) provides the strongest and clearest signal, (Fig.  
 577 S4E), and genes active in this tissue are mainly linked to enzymatic activity  
 578 (Table S4). Such genes are usually activated by mating and show a decreased  
 579 response in monogamous females (Fig. S4A). Significant genes in the ovaries  
 580 (module 5 and 6) are involved in gamete production, while in the neural tissues  
 581 they regulate signaling processes and transmembrane transport activity (Table  
 582 S5, S6). The last category (module 2 and 3) contains genes expressed in a diverse  
 583 array of tissues (Table 2), although known to show overall very high correlation  
 584 for expression (Innocenti & Morrow, 2010). The Gene Ontology categories  
 585 involved (oxidoreductase activity, lipid and sugars storage/metabolism, Table  
 586 S2, S3) seem to indicate a predominant function in energy production and  
 587 resource consumption. An additional, small category points to transcripts mostly  
 588 active in the testes, and its interpretation is problematic, given the sex-limited  
 589 nature of this tissue. This set of genes, however, which are not involved in a  
 590 normal post-mating reaction (Fig. S1A), could be selected due to pleiotropic  
 591 activity in other tissues, or exhibit non-random segregation (e.g. linkage  
 592 disequilibrium) with selected transcripts.

593

594 In *D. melanogaster*, transfer of the ejaculate, and in particular some seminal fluid  
 595 components (e.g. Sex Peptide), radically transforms female behaviour and  
 596 physiology, leading to increased egg production (Gillott, 2003), decreased  
 597 receptivity (Chapman *et al.*, 2003) and increased feeding behaviour (Carvalho *et*  
 598 *al.*, 2006), and has profound effects on gene expression, especially gene products  
 599 affecting metabolic rate (Innocenti & Morrow, 2009). Moreover, at least part of  
 600 these physiological changes are mediated by the nervous system (Hasemeyer *et*  
 601 *al.*, 2009). Together this diverse range of evidence confirms that monogamous  
 602 females are less fecund than promiscuous females because they exhibit a weaker

post-mating response. The origin of this difference in response might reflect a lower 'genetic quality' of monogamous females arising from a relaxed selection on female resistance to male harassment or from relaxed post-copulatory selection, which can directly affect female ability to induce a physiological response to mating, or be mediated by an overall weaker condition. In this respect, Fricke *et al.* (2010) recently showed how nutritional status of females determines their response to the sex peptide and influences fecundity, with high food diets being associated with increased egg production, raising the hypothesis that female response to mating can be environmental or condition dependent.

This study, in combination with the independent characterization of post-mating expression profiles in females and the relationship between transcript abundance and female fitness in the ancestral population, provides a robust list of candidate genes associated with changes in female fecundity caused by evolution under different sexual selective pressures. Given the close agreement between what is already known about the effects of the male ejaculate on females and their fitness in *D. melanogaster*, with the general patterns of tissue specificity and biological processes identified here, these data provide a clear indication as to which genes are the targets of post-mating sexual selection in this promiscuously mating population.

## Acknowledgments

We thank Jessica Abbott, Tim Connallon and Björn Rogell for comments on the manuscript and the Uppsala Array Platform. Funding was provided by the Swedish Research Council (Grant #2008-5533 and #2011-3701), the European Research Council (Grant #280632) and a Royal Society University Research Fellowship to EHM.

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816

817

# Figure legends

Figure 1. *Body size*. Wing length of females and males evolved under monogamous (M) and promiscuous (P) mating systems.

Figure 2. *Female fecundity*. Reproductive output of females evolved under monogamous and promiscuous selection regimes after mating once (panels B and C) or being continuously exposed to males (panels A and D), during the normal reproductive window (panel A and B) or during a longer interval (4 days; panel C and D).

Figure 3. *Reversed selection*. Reproductive output of females evolved under monogamous (M), monogamous then promiscuous (MP) and promiscuous (P) selection regimes. Results of the posthoc analysis are given above the plotting frame, letters not shared indicate treatments that show statistical significant differences (see Results for details).

Figure 4. *Association with post-mating response and female fitness*. (A) Density distribution of significant up-regulated (blue) and down-regulated (red) transcripts along all the tested genes, ranked according to their post-mating reaction (data from a previously published study on the same population; Innocenti and Morrow, 2009). (B) Density distribution of the significant transcripts along all the tested genes, ranked by the t-value of their association with female fitness (data from a previously published study on the same population; Innocenti and Morrow, 2010).

Figure 5. *Transcriptional modules*. Level-plot representing the matrix of pair-wise correlation for the expression of the 1141 significant transcripts across tissues of *D. melanogaster* (data from Chintapalli *et al.*, 2007). The correlation matrix has been used to compute modules of correlated expression (separated by grey lines). The 7 modules containing more than 50 genes are labeled.

850

**Tables**

851

	<b>Estimate</b>	<b>Sum Square</b>	<b>d.f.</b>	<b>F<sub>1,13</sub></b>	<b>P</b>
<b>Trial 1</b>					
Intercept	409.2	2679442	1	339.15	<0.001
Female type	-74.7	89401	1	11.32	0.0051
Male type	22.9	8372	1	1.06	0.3221
Residuals		102707	13		
<b>Trial 2</b>					
Intercept	563.6	5081418	1	1709.80	<0.001
Female type	-30.2	14544	1	4.89	0.0455
Male type	-18.5	5476	1	1.84	0.1977
Residuals		38635	13		
<b>Trial 3</b>					
Intercept	1119.3	20044424	1	3353.02	<0.001
Female type	-47.6	36214	1	6.06	0.0286
Male type	9.4	1406	1	0.24	0.6357
Residuals		77714	13		
<b>Trial 4</b>					
Intercept	1707.2	46633192	1	2016.38	<0.001
Female type	-65.1	67834	1	2.93	0.1105
Male type	-13.5	2932	1	0.13	0.7275
Residuals		300654	13		

852

853 **Table 1. Linear model results on female fecundity.** *Trial 1:* Females  
854 continuously exposed to males, 18 h oviposition period; *Trial 2:* Females mated  
855 once, 18 h oviposition period; *Trial 3:* Females mated once, 4 days oviposition  
856 period; *Trial 4:* Females continuously exposed to males, 4 days oviposition  
857 period.

Module	Size	Tissue	Up	Down	Fitness assoc.	GO terms
1	55	Testes	24 (n.s.)	31 (n.s.)	Yes (0.016)	Table S1
2	158	Carcass, Head, Eyes, Heart, Fat body, Spermatheca	52 (mixed, <0.001)	106 (up, <0.001)	Yes (<0.001)	Table S2
3	108	Crop, Mid gut, Hind gut, Heart, Fat body, Spermatheca	42 (mixed, 0.007)	66 (n.s.)	Yes (<0.001)	Table S3
4	124	Mid gut	33 (mixed, 0.002)	91 (up, <0.001)	Yes (<0.001)	Table S4
5	129	Ovary	83 (down, <0.001)	46 (n.s.)	Yes (0.006)	Table S5
6	164	Brain, Ovary	113 (n.s.)	51 (down, 0.002)	Yes (0.014)	Table S6
7	105	Brain, Eyes, Head, Thoracic ganglion	25 (n.s.)	80 (up, <0.001)	Yes (0.047)	Table S7

**Table 2. Summary description of the main modules.** ‘Size’: Number of significant genes in each module (n>50). ‘Tissue’: tissues in which the transcripts in each module are significantly over-expressed compared to the whole body (data from Chintapalli *et al.*, 2007). ‘Up’ (‘Down’) is the subset of up-regulated (down-regulated) transcripts in the module. In parentheses is indicated whether the subset tend to be up-regulated or down-regulated after mating, or a mix of the two (mixed); P value from a MR-GSE test; see also Figs.S1A-S7A, (data from Innocenti & Morrow, 2009). ‘Fitness assoc.’ indicates whether the genes in the module are over-represented among the genes found to be associated with female fitness (MR-GSE test, data from Innocenti & Morrow, 2010).











